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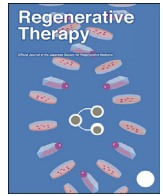
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## Original Article

# Enhanced survival and insulin secretion of insulinoma cell aggregates by incorporating gelatin hydrogel microspheres

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## ABSTRACT

**Introduction:** The objective of this study is to evaluate the survival and glucose-induced insulin secretion of rat-derived insulinoma cells (INS-1) from their aggregates incorporating different size of gelatin hydrogel microspheres comparing with microspheres-free cell aggregates.

**Methods:** The gelatin hydrogel microspheres were prepared by the conventional w/o emulsion method. The INS-1 cells were cultured in a V-bottomed well, combining with or without the gelatin hydrogel microspheres to form their aggregates with or without microspheres.

**Results:** When the cell viability, the live cell number, the reductase activity, and the insulin secretion of cell aggregates were evaluated 7 or 14 days after incubation, the cell aggregates incorporating gelatin hydrogel microspheres showed higher cell viability, reductase activity and a larger number of live cells. The cell aggregates incorporating larger size and number of gelatin hydrogel microspheres secreted a larger amount of insulin, compared with those incorporating smaller size and number of microspheres or without microspheres.

**Conclusion:** It is conceivable that the incorporation of gelatin hydrogel microspheres in cell aggregates is promising to improve their survival and insulin secretion function.

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## 1. Introduction

Islet transplantation has been investigated as a treatment of type 1 diabetes for patients with insufficient glucose control [1–3]. However, a big problem of islet transplantation therapy is the serious donor shortage [4–6]. To circumvent this issue, it has been reported to reconstitute islet-like aggregates of insulin secreting cells [7,8]. However, for this approach, when the cell aggregates become larger than 200  $\mu$ m in diameter, the cells in the center of cell aggregates tend to die because of a lack of oxygen and nutrients supply [9,10]. It is well known that insulin secreting cells show a decreased function of insulin secretion under a hypoxic

environment [11,12]. Therefore, to achieve sufficient therapeutic effect with the insulin secreting cell aggregates, it is necessary to develop a method for the promotion of oxygen and nutrients supply.

Previous studies demonstrated that the incorporation of gelatin hydrogel microspheres in mesenchymal stem cells (MSC) aggregates enabled the cells to improve the viability, proliferation and osteogenic differentiation. This is because the microspheres improved the state of oxygen and nutrients supply for cells [13,14]. In this study, the gelatin hydrogel microspheres technology was introduced to insulin secreting cell aggregates to assess the cell viability and insulin secretion function comparing with microspheres-free cell aggregates. Gelatin hydrogel microspheres with different sizes were prepared by the conventional w/o emulsion method previously reported [15]. Rat insulinoma cells (INS-1), the model of insulin secreting cells, were incubated with or without the gelatin hydrogel microspheres in a V-bottomed well to form the cell aggregates with or without the microspheres. We examined the effect of microspheres size and number on the cell viability, reductase activity, and insulin secretion ability in the aggregates.

**Abbreviations:** INS-1, insulinoma; MSC, mesenchymal stem cell.

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## 2. Materials and methods

### 2.1. Preparation of gelatin hydrogel microspheres

Gelatin hydrogel microspheres were prepared by the chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported [15]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoionic point 5.0 (pI 5), weight-averaged molecular weight = 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 40 °C, followed by stirring at 200 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked microspheres. The resulting microspheres were washed three times with cold acetone in combination with centrifugation (5000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20, 32, and 53 µm (Iida Seisakusyo Ltd., Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried gelatin microspheres (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for 48 h for the dehydrothermal crosslinking of gelatin. Pictures of gelatin hydrogel microspheres in a dispersed state in RPMI medium 1640 containing L-glutamine (Invitrogen Ltd., Carlsbad, CA), were taken with a light microscope (BZ-X710, KEYENCE Corp., Osaka, Japan). The size of 100 microspheres for each sample was measured using the computer program of microscope (BZ-X710) to calculate the average diameter.

### 2.2. Preparation of INS-1 cell aggregates with or without gelatin hydrogel microspheres

A cell line 832/13, derived from INS-1 rat insulinoma cells, was obtained from Dr. Christopher B. Newgard (Duke University Medical Center, Durham, NC) [16]. Cells were grown in RPMI medium 1640 containing L-glutamine (Invitrogen Ltd.), 1 mM sodium pyruvate (Invitrogen Ltd.), 10 mM HEPES (Invitrogen Ltd.), 10 vol% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA), 55 µM 2-mercaptoethanol (Invitrogen Ltd.), 100 IU/ml penicillin (Gibco, Grand Island, NY), and 100 µg/ml streptomycin (Gibco). Cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air at 37 °C.

Gelatin hydrogel microspheres and INS-1 cells were separately suspended in the culture medium under different conditions (Table 1). Gelatin microsphere suspensions (100 µl) were added to each well of a 96-well culture plate with V-bottomed wells, followed by 50 µl of INS-1 cell suspensions at the initial density of  $1.0 \times 10^3$  or  $1.0 \times 10^4$  cells/well and  $1.0 \times 10^1$ ,  $0.5 \times 10^2$ ,  $1.0 \times 10^2$  or

$1.0 \times 10^3$  microspheres/well. The cells/microspheres number ratio is 10/1, 100/1, or 200/1. Pictures of INS-1 cell aggregates with or without gelatin hydrogel microspheres were taken with the microscope as described above.

### 2.3. Evaluation of cell viability, reductase activity, and live cell number

The number of live cells in INS-1 cell aggregates with or without gelatin hydrogel microspheres was determined by trypan-blue staining. After 7 or 14 days incubation, cell aggregates were collected into a 1.5 ml microtube and washed by 100 µl of phosphate-buffered saline solution (PBS, Gibco) once. After centrifugation and the supernatants removal, the aggregate pellets were incubated in 100 µl of trypsin–EDTA solution (Sigma–Aldrich Company Ltd., St. Louis, MO) for 15 min at 37 °C. Following the incubation, 100 µl of culture medium was added to stop the trypsin action. Live cells in the cell suspension were counted by a cell counter (Countess II FL, Thermo Fisher Scientific Inc.). Live/dead assays were conducted using a Live/Dead Viability/Cytotoxicity Assay (Invitrogen) according to the manufacturer's protocol. The cell aggregates were rinsed once with PBS 7 or 14 days after incubation, and then incubated with the mixed solution of 2 mM calcein AM and 4 mM ethidium homodimer-1 solution in PBS for 15 min at room temperature in the dark, followed by observation using a microscope (BX-X710).

Reductase activity of INS-1 cell aggregates with or without gelatin hydrogel microspheres was determined by colorimetric assay using water-soluble tetrazolium salt as one measure to assess a cell metabolic activity. After 7 or 14 days incubation, cell aggregates were collected into a well of 96 well flat-bottomed plate and the culture medium was adjusted 100 µl in each well. WST-8 reagent (10 µl, Dojindo Laboratories, Kumamoto, Japan) was added into each well, and then cell aggregates were incubated for 4 h at 37 °C. After the incubation, the absorbance at 450 nm was measured by SpectraMax M2/M2e Microplate Reader (Molecular Devices, LLC, CA). The reductase activity was normalized by the live cell number.

### 2.4. Evaluation of glucose-induced insulin secretion

After 7 or 14 days incubation, INS-1 cell aggregates with or without gelatin hydrogel microspheres were transferred to each well of a 12 mm Transwell (#3402, Corning Inc. Corning, NY) and washed once by PBS. Then, Krebs-Ringer-bicarbonate HEPES (KRB) buffer solution containing 10 mM glucose [16] was added to each well, and the cell aggregates were incubated for 1 h at 37 °C. The concentration of secreted insulin in the supernatants was measured by ELISA kit (Rat Insulin ELISA KIT, Shibayagi Co. Ltd., Gunma, Japan). The insulin secretion was normalized by the live cell number.

### 2.5. Statistical analysis

All the statistical data are expressed as mean ± standard error of the mean (SEM). The data were analyzed using the Tukey's test and the statistical significance was accepted at  $P < 0.05$  or  $0.01$ .

## 3. Result

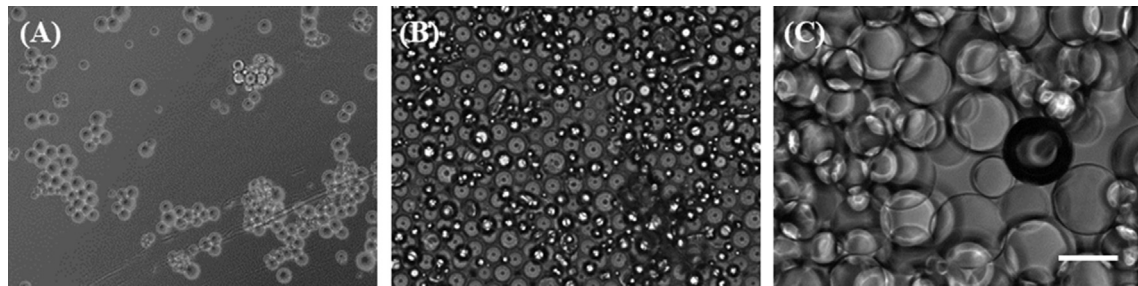
### 3.1. Characterization of gelatin hydrogel microspheres

Fig. 1 shows the microscopic images of gelatin hydrogel microspheres. The microspheres had spherical shape and smooth surface.

**Table 1**

Preparation conditions of INS-1 cells aggregate with or without gelatin microspheres incorporation

Code	Number of INS-1 cells (cells/well)	Number of gelatin hydrogel microspheres (/well)	Size of gelatin hydrogel microspheres (mm)
a	$1.0 \times 10^3$	0	—
b	$1.0 \times 10^3$	$1.0 \times 10^1$	$46.0 \pm 14.0$
c	$1.0 \times 10^3$	$1.0 \times 10^1$	$82.0 \pm 18.0$
d	$1.0 \times 10^4$	0	—
e	$1.0 \times 10^4$	$1.0 \times 10^2$	$22.0 \pm 9.0$
f	$1.0 \times 10^4$	$1.0 \times 10^3$	$22.0 \pm 9.0$
g	$1.0 \times 10^4$	$1.0 \times 10^2$	$46.0 \pm 14.0$
h	$1.0 \times 10^4$	$0.5 \times 10^2$	$82.0 \pm 18.0$
i	$1.0 \times 10^4$	$1.0 \times 10^2$	$82.0 \pm 18.0$



**Fig. 1.** Light microscopic pictures of gelatin hydrogel microspheres dispersed in the culture medium. The diameter of gelatin hydrogel microspheres is  $22.0 \pm 9.0$  (small) (A),  $46.0 \pm 14.0$  (middle) (B) or  $82.0 \pm 18.0$   $\mu\text{m}$  (large) (C). Scale bar is 100  $\mu\text{m}$ .

The size of microspheres in the swollen condition in the culture medium was  $22.0 \pm 9.0$  (small),  $46.0 \pm 14.0$  (middle) and  $82.0 \pm 18.0$   $\mu\text{m}$  (large).

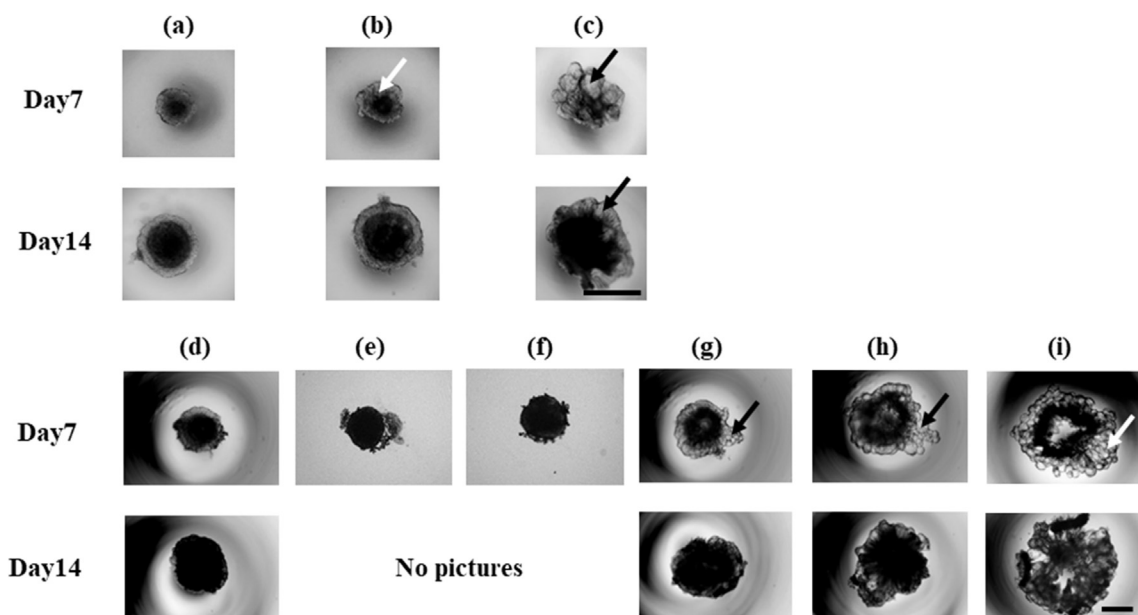
### 3.2. Characterization of INS-1 aggregates with and without gelatin hydrogel microspheres

Table 1 shows the preparation condition of cell aggregate using middle or large microspheres. Small gelatin hydrogel microspheres with the diameter of  $22.0 \pm 9.0$   $\mu\text{m}$  were used for INS-1 cell aggregate preparation. However, the appearance of cell aggregate was similar to that of cell aggregate without microspheres (Fig. 2 (d)–(f)). Based on this, middle and large gelatin hydrogel microspheres were used for the formation of cell aggregates unless otherwise mentioned. Fig. 2 shows the microscopic pictures of INS-1 cell aggregates 7 or 14 days after incubation. Cell aggregates were successfully formed in V-bottomed wells. Cell aggregates had a fragile structure when they were prepared with larger size and number of microspheres at the initial seeding density of  $1.0 \times 10^4$  cells/well even 14 days after incubation (Fig. 2 (i)). On the other hand, when the initial seeding density was  $1.0 \times 10^3$  cells/well, cell aggregates were successfully obtained even in the case of larger size microspheres.

### 3.3. Effect of gelatin hydrogel microspheres on cell viability and reductase activity in cell aggregates

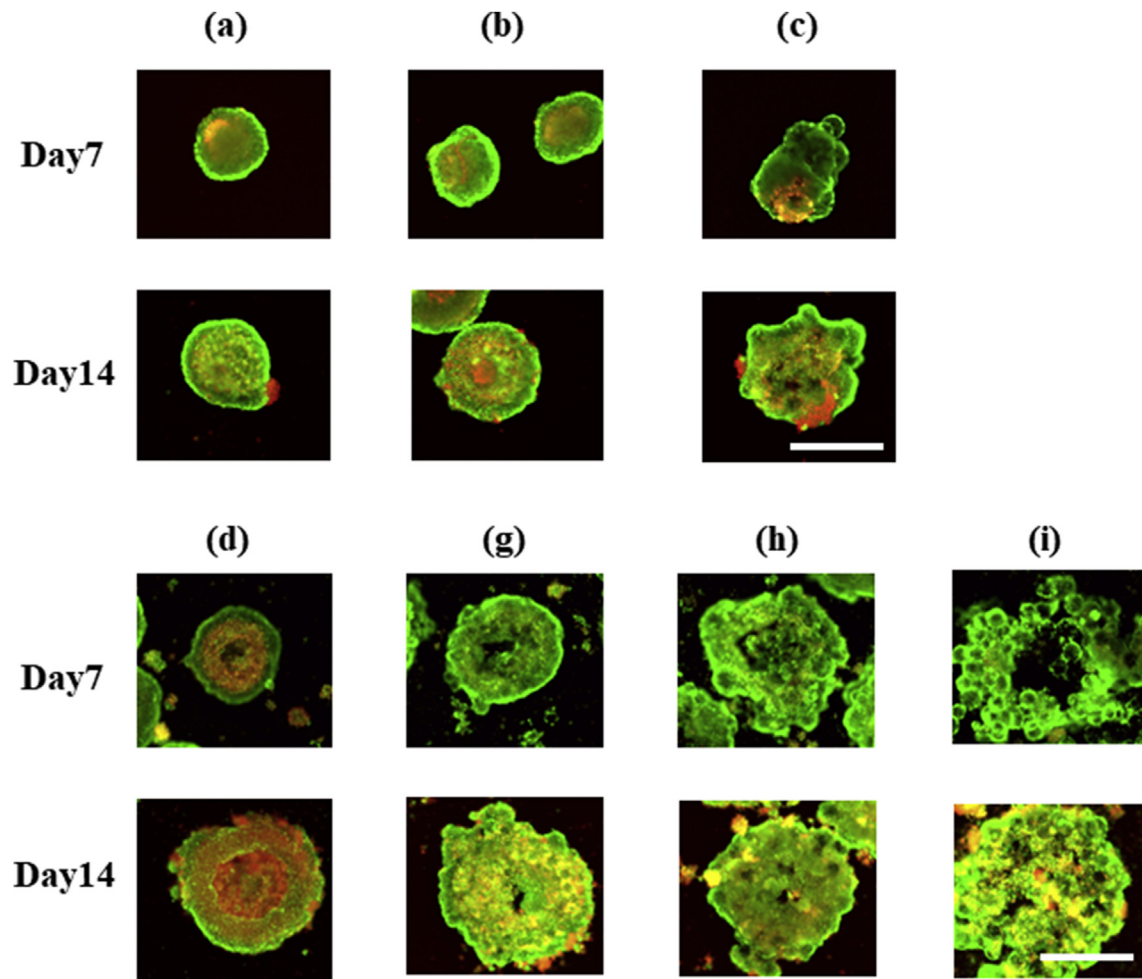
Fig. 3 shows the fluorescent images of cell aggregates after the live/dead assay. To assess the cell viability in INS-1 cell aggregates, the live/dead assay was conducted. At the initial seeding density of  $1.0 \times 10^4$  cells/well, the cells in the center of microspheres-free cell aggregates died (Fig. 3 (d)). On the other hand, the microspheres suppressed the cell death (Fig. 3 (g)–(i)), irrespective of the size and number of microspheres incorporated. The microspheres incorporation did not affect the cell viability in cell aggregates at the initial seeding density of  $1.0 \times 10^3$  cells/well (Fig. 3 (a)–(c)).

Trypan-blue exclusion test was performed to evaluate the number of live cells in cell aggregates. Fig. 4 shows the number of live cells 7 or 14 days after incubation. The number of live cells showed little change for cell aggregates prepared at the initial seeding density of  $1.0 \times 10^3$  cells/well with incubation time. On the other hand, the number decreased for cell aggregates at the initial seeding density of  $1.0 \times 10^4$  cells/well. For cell aggregates at the initial seeding density both of  $1.0 \times 10^3$  and  $1.0 \times 10^4$  cells/well, the number of live cells in cell aggregates incorporating large size and number of gelatin hydrogel microspheres was significantly larger than that of cell aggregates incorporating middle microspheres and without microspheres 7 days after incubation.

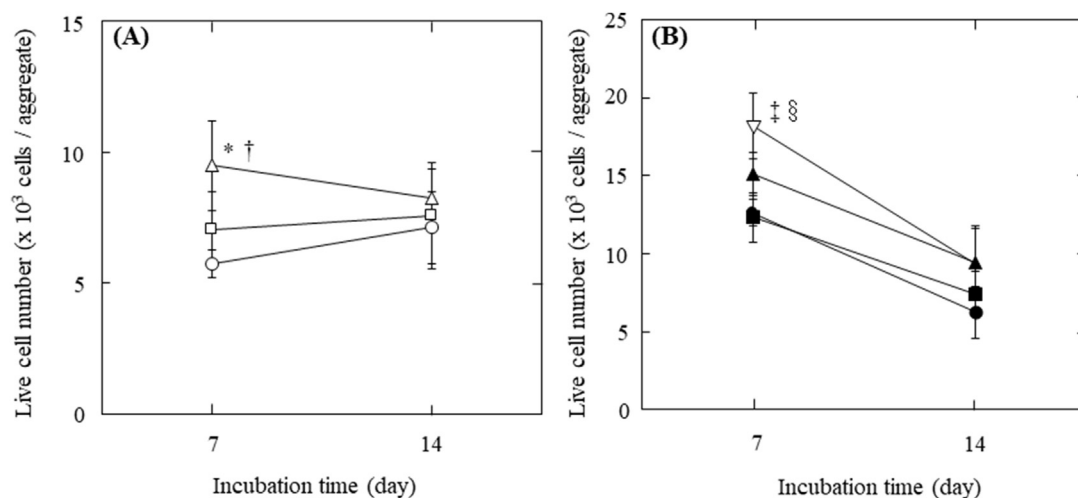


**Fig. 2.** Light microscopic pictures of INS-1 cell aggregates 7 and 14 days after incubation with or without gelatin hydrogel microspheres. Symbols (a)–(i) correspond to those in Table 1. Arrows indicate gelatin hydrogel microspheres. Scale bar is 500  $\mu\text{m}$ .





**Fig. 3.** Live/dead assay pictures of INS-1 cell aggregates with or without gelatin hydrogel microspheres 7 and 14 days after incubation. Symbols (a)–(i) correspond to those in Table 1. Scale bar is 500  $\mu$ m.



**Fig. 4.** Live INS-1 cell number per aggregate with or without gelatin hydrogel microspheres 7 and 14 days after incubation. (A) Cell aggregates with  $1.0 \times 10^1$  of middle size ( $\square$ ) and large size gelatin hydrogel microspheres ( $\triangle$ ) or without gelatin hydrogel microspheres ( $\circ$ ) at the initial seeding density of  $1.0 \times 10^3$  cells/well. \* $p < 0.01$ , significant against the number of live INS-1 cells in cell aggregate without gelatin hydrogel microspheres at the corresponding time;  $\dagger p < 0.05$ , significant against the number of live INS-1 cells with middle size of gelatin hydrogel microspheres at the corresponding time. (B) Cell aggregates with  $1.0 \times 10^2$  of middle size ( $\blacksquare$ ),  $0.5 \times 10^2$  of large size ( $\blacktriangle$ ) and  $1.0 \times 10^2$  of large size of gelatin hydrogel microspheres ( $\nabla$ ) or without gelatin hydrogel microspheres ( $\bullet$ ) at the initial seeding density of  $1.0 \times 10^4$  cells/well.  $\ddagger p < 0.01$ , significant against the number of live INS-1 cells in cell aggregate without gelatin hydrogel microspheres at the corresponding time;  $\S p < 0.01$ , significant against the number of live INS-1 cells with middle size of gelatin hydrogel microspheres at the corresponding time.

Fig. 5 shows the reductase activity per INS-1 cell aggregate or INS-1 cell 7 and 14 days after incubation with or without gelatin hydrogel microspheres. The reductase activity per aggregate increased with incubation time. For cell aggregates at the initial seeding density of  $1.0 \times 10^3$  and  $1.0 \times 10^4$  cells/well, the activity per aggregate incorporating large size and number of gelatin hydrogel microspheres tended to be larger than that of cell aggregate incorporating middle microspheres and without microspheres (Fig. 5A and B). The profile of the reductase activity per cell was the similar to that per aggregate (Fig. 5C and D).

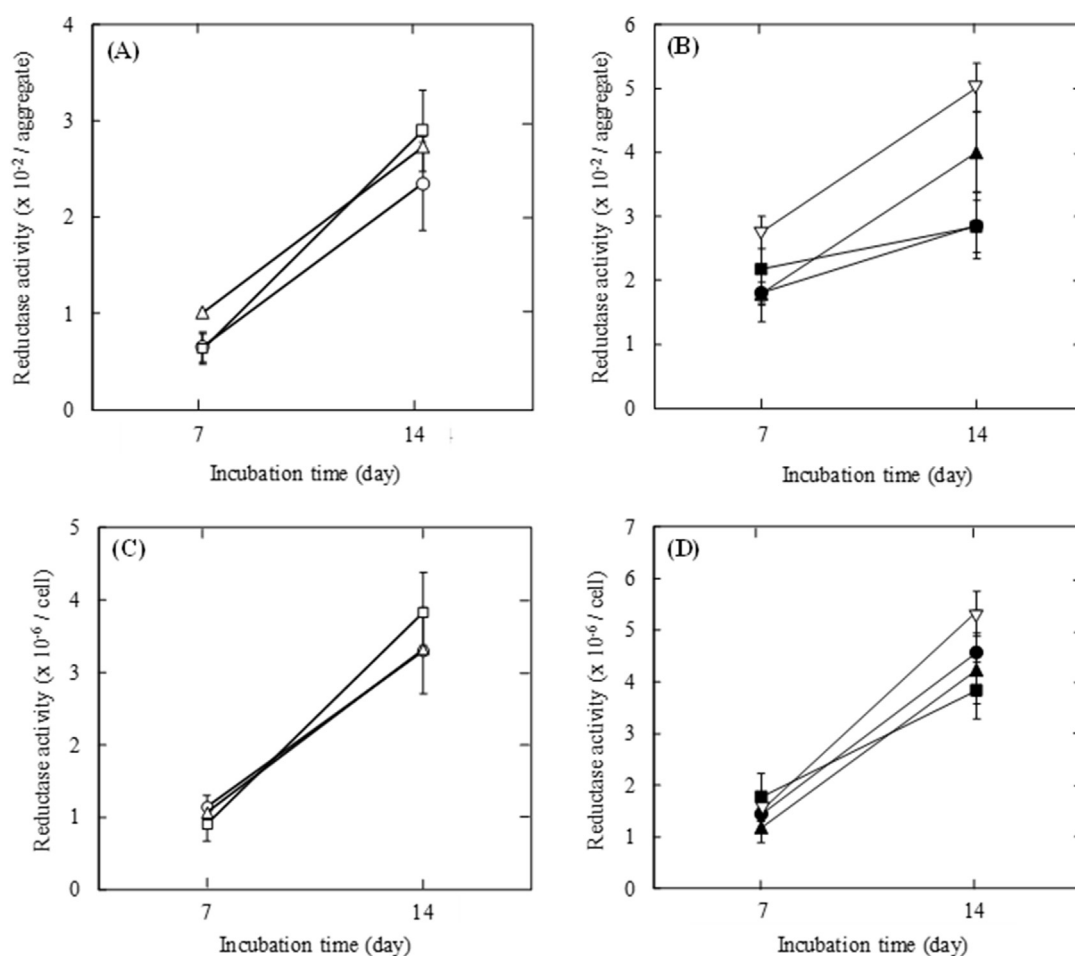
#### 3.4. Effect of gelatin hydrogel microspheres on glucose-induced insulin secretion of cell aggregates

Fig. 6 shows the insulin secretion per INS-1 cell aggregate or INS-1 cell 7 and 14 days after incubation with or without gelatin hydrogel microspheres. Cell aggregates with large microspheres secreted insulin at significantly larger amount than those with middle microspheres and without microspheres for 14 days at the initial seeding density of  $1.0 \times 10^3$  cells/well. On the other hand, at the initial seeding density of  $1.0 \times 10^4$  cells/well, cell aggregates with large microspheres tended to secrete insulin at larger amount than those with middle microspheres and without microspheres,

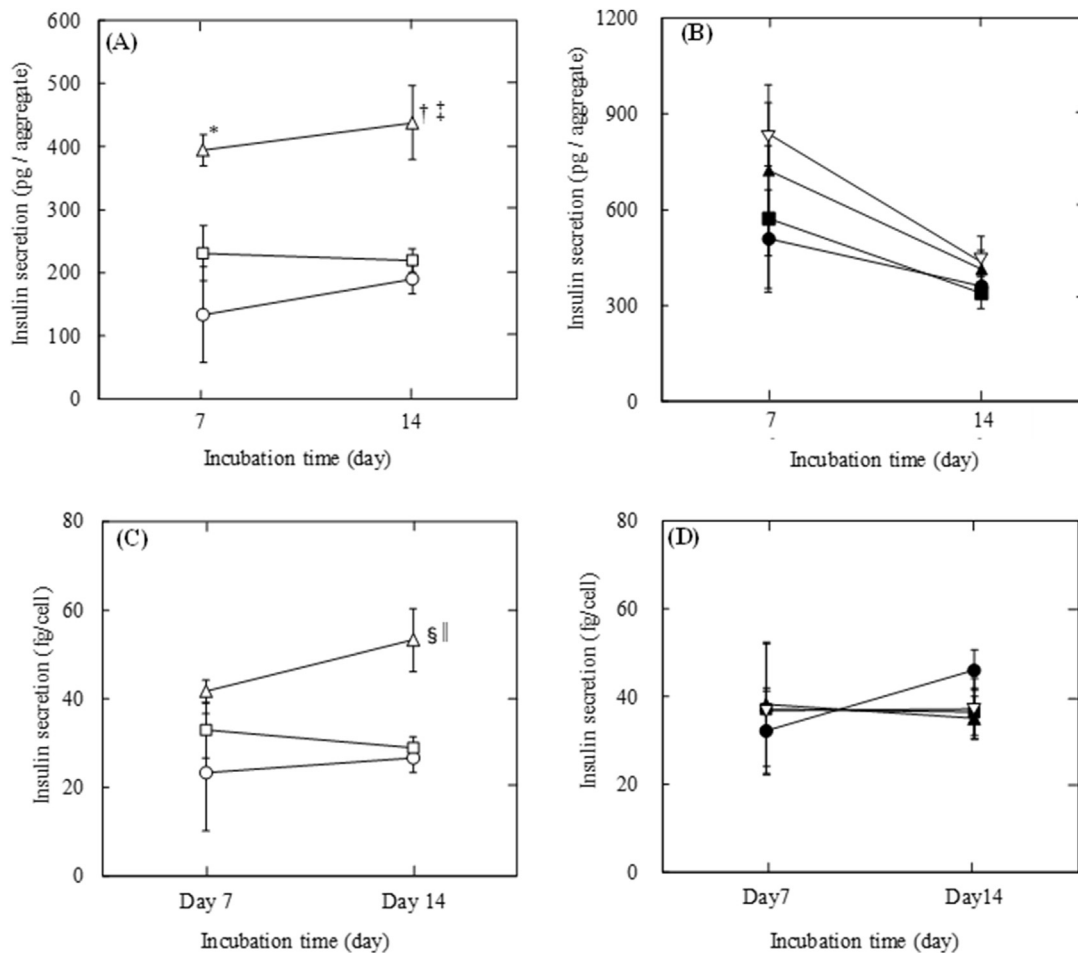
and the decreasing tendency of insulin secretion was observed (Fig. 6A and B). The insulin secretion per cell in aggregate incorporating large microspheres was also significantly higher than that in aggregate with middle microspheres and without microspheres at 14 days after incubation at the initial seeding density of  $1.0 \times 10^3$  cells/well. However, at the initial seeding density of  $1.0 \times 10^4$  cells/well, there was no difference among cells in each cell aggregate (Fig. 6C and D).

#### 4. Discussion

Generally, U and V-bottomed multi-well plates or a hanging-drop method are used to prepare cell aggregates in lab-scale [17–20]. However, the aggregates formation depended on the type of cells. For example, INS-1 cell aggregates did not form when 96-well U-bottomed or Flat-bottomed plate was used. This is because cells adhered to the bottom surface or wall of well unless the well was coated for anti-cell adhesion (data not shown) [13,14]. On the other hand, cell aggregates were obtained in the V-bottomed well even without the anti-cell adhesion coating. It is reported that cells did not remain on the wall of wells because the V-bottomed well had too steep wall to induce cell adhesion [20]. The formation of cell aggregate depended on the size and number



**Fig. 5.** Reductase activity 7 and 14 days after incubation. (A) Reductase activity of INS-1 cell aggregate with  $1.0 \times 10^3$  of middle size ( $\square$ ) and large size gelatin hydrogel microspheres ( $\triangle$ ) or without gelatin hydrogel microspheres ( $\circ$ ) at the initial seeding density of  $1.0 \times 10^3$  cells/well. (B) Reductase activity of INS-1 cell aggregate with  $1.0 \times 10^2$  of middle size ( $\blacksquare$ ),  $0.5 \times 10^2$  of large size ( $\blacktriangle$ ) and  $1.0 \times 10^2$  of large size of gelatin hydrogel microspheres ( $\nabla$ ) or without gelatin hydrogel microspheres ( $\bullet$ ) at the initial seeding density of  $1.0 \times 10^4$  cells/well. (C) Reductase activity of INS-1 cell in aggregates with  $1.0 \times 10^3$  of middle size ( $\square$ ) and large size gelatin hydrogel microspheres ( $\triangle$ ) or without gelatin hydrogel microspheres ( $\circ$ ) at the initial seeding density of  $1.0 \times 10^3$  cells/well. (D) Reductase activity of INS-1 cell in aggregates with  $1.0 \times 10^2$  of middle size ( $\blacksquare$ ),  $0.5 \times 10^2$  of large size ( $\blacktriangle$ ) and  $1.0 \times 10^2$  of large size of gelatin hydrogel microspheres ( $\nabla$ ) or without gelatin hydrogel microspheres ( $\bullet$ ) at the initial seeding density of  $1.0 \times 10^4$  cells/well.



**Fig. 6.** Insulin secretion 7 and 14 days after incubation. (A) Insulin secretion per INS-1 cell aggregate with  $1.0 \times 10^1$  of middle size ( $\square$ ) and large size gelatin hydrogel microspheres ( $\triangle$ ) or without gelatin hydrogel microspheres ( $\circ$ ) at the initial seeding density of  $1.0 \times 10^3$  cells/well. \*p < 0.05,  $\dagger$ p < 0.01, significant against insulin secretion per aggregate without gelatin hydrogel microspheres at the corresponding time.  $\ddagger$ p < 0.05, significant against insulin secretion per aggregate with middle size of gelatin hydrogel microspheres at the corresponding time. (B) Insulin secretion per INS-1 cell aggregate with  $1.0 \times 10^2$  of middle size ( $\blacksquare$ ),  $0.5 \times 10^2$  of large size ( $\blacktriangle$ ) and  $1.0 \times 10^2$  of large size of gelatin hydrogel microspheres ( $\nabla$ ) or without gelatin hydrogel microspheres ( $\bullet$ ) at the initial seeding density of  $1.0 \times 10^4$  cells/well. (C) Insulin secretion per INS-1 cell in aggregates with  $1.0 \times 10^3$  of middle size ( $\square$ ) and large size gelatin hydrogel microspheres ( $\triangle$ ) or without gelatin hydrogel microspheres ( $\circ$ ) at the initial seeding density of  $1.0 \times 10^3$  cells/well.  $\S$ p < 0.05, significant against insulin secretion per aggregate without gelatin hydrogel microspheres at the corresponding time.  $\parallel$ p < 0.05, significant against insulin secretion per aggregate with middle size of gelatin hydrogel microspheres at the corresponding time. (D) Insulin secretion per INS-1 cell in aggregates with  $1.0 \times 10^2$  of middle size ( $\blacksquare$ ),  $0.5 \times 10^2$  of large size ( $\blacktriangle$ ) and  $1.0 \times 10^2$  of large size of gelatin hydrogel microspheres ( $\nabla$ ) or without gelatin hydrogel microspheres ( $\bullet$ ) at the initial seeding density of  $1.0 \times 10^4$  cells/well.

of microspheres (Fig. 2). Cell aggregates were not completely formed upon using larger size and number of microspheres even 14 days after incubation at the initial seeding density of  $1.0 \times 10^4$  cells/well. It is likely that microspheres tended to sink down to the well bottom faster than cells because of their heavy weight, and larger number of microspheres ( $0.5$  or  $1 \times 10^2$  microspheres/well) tended to occupy the larger volume of the well bottom. Consequently, the microspheres were firstly gathered on the bottom surface of wells and then cells surrounded them (Supplemental Fig. 1), because cells could not sufficiently go through the spaces inbetween larger number of microspheres. It is highly conceivable that heterogeneous distribution of cells and microspheres structurally weakened cell aggregates. This result suggests that there is an optimal number ratio of cells to microspheres.

In this study, gelatin was used for the material of hydrogel microspheres because of its high chemical susceptibility and biocompatibility. Gelatin is biodegradable material and has been extensively used for food, pharmaceutical, and medical purposes and its biosafety has been proven through their long practical applications [21–25]. However, for the formation of more rigid cell

aggregates, it is required to modify or optimize the hydrogel microspheres to promote INS-1 cell attachment to the surface of microspheres. The coating of cell adhesion proteins, such as fibronectin, laminin, vitronectin, and collagen, is known to improve cell adhesion to the surface of microspheres [26–28]. Isoionic point 9.0 (pI 9) gelatin has a positive charge at the physiological pH and might promote cell adhesion because cell surfaces have a negative charge [29,30]. We evaluated INS-1 cell attachment to the surface of low-attachment 24 well plate (#3473, Corning Inc.) which had been coated by gelatin of pI5 or 9 (weight-averaged molecular weight  $\approx 1,00,000$ , Nitta Gelatin Inc.), collagens, and cell adhesion proteins. The concentration of coating solutions and coating methods were decided according to the manufacturer's protocol. The protocol for the coating of gelatin (pI 5 or 9) referred to that of collagen. The gelatin of pI9, collagen type 1 (Type I-A, I-P; Nitta Gelatin Inc./I-AC, Koken Co., Ltd., Tokyo), type 3 (PSC-3-100-05; Nippi Inc., Tokyo, Japan), or type 4 (ASC-4-104-01; Nippi), fibronectin (Wako Pure Chemical Industries Ltd.), and vitronectin (Sigma–Aldrich Company Ltd.) improved the INS-1 cells attachment compared with gelatin of pI 5 (Supplemental Fig. 2). On the other

hand, laminin (Wako Pure Chemical Industries Ltd.) did not promote the cell attachment. Taken together, the surface coating of gelatin hydrogel microsphere by collagen, fibronectin or vitronectin might be promising to improve cell attachment and form more rigid aggregates, although the coating onto the surface of tissue culture plates does not represent directly that of gelatin hydrogel microspheres.

The viability of cells in the center of cell aggregates without microspheres was obviously bad when the initial seeding density was  $1.0 \times 10^4$  cells/well even 7 days after incubation (Fig. 3). On the other hand, it is apparent that the microspheres incorporation suppressed the cell death, although a little decrease in the cell viability was detected, because the aggregates showed yellow color which were mixed with green live cells and red dead cells. The number of live cells in cell aggregates was improved by the incorporation of gelatin hydrogel microspheres, especially for larger size and number of microspheres (Fig. 4). It is demonstrated that oxygen and nutrients did not supply sufficiently to cells present in the center of aggregates without microspheres [9,10]. We previously demonstrated that the incorporation of gelatin hydrogel microspheres enabled MSC in their aggregates to improve the cell viability and proliferation, and osteogenic differentiation [13,14]. We can say with certainty that the gelatin hydrogel microspheres gave a pathway of oxygen to the cell aggregates. Especially, larger size and number of microspheres would give more efficient pathway of oxygen to cells inside the cell aggregates than smaller size and number of microspheres. Therefore, a large number of live cells would remain in the cell aggregates incorporating larger size and number of microspheres. As seen in Fig. 4B, the live cell number was notably decreased with the incubation time when the initial seeding density was  $1.0 \times 10^4$  cells/well. In addition, the cell number only slightly increased compared with the cell number seeded initially, whereas the cell number increased 5–10 folds compared with the initial cell number at the initial seeding density of  $1.0 \times 10^3$  cells/well. The initial seeding density of  $1.0 \times 10^4$  cells/well would be too high to allow cell aggregates to grow in the V-bottomed well. It is conceivable that the insufficient space physically hindered the cell proliferation. Taken together, this result suggests that there must be an optimal seeding density for each culture substrate not to physically damage the cell aggregates.

It is apparent from Fig. 2 that cell number increased with incubation time for cell aggregates at the initial seeding density of  $1.0 \times 10^3$  cells/well (a–c), aggregates without microspheres (d) and with middle microspheres (e) at the initial seeding density of  $1.0 \times 10^4$  cells/well, because the size of aggregate increased with incubation time. The aggregates with larger microspheres at the initial seeding density of  $1.0 \times 10^4$  cells/well (h, i) did not increase the size, but the structural density seems to become higher with the incubation time. We can think that this is because the increasing cell number resulted in filling the spaces between aggregates. However, trypan-blue staining showed that live cell number did not increase at the initial seeding density of  $1.0 \times 10^3$  cells/well, and live cell number decreased at that of  $1.0 \times 10^4$  cells/well (Fig. 4). This implies that the lack of oxygen and nutrients caused the necrosis of cells in the center of aggregate, and the narrow bottom space of well damaged cells in aggregate. At the initial seeding density of  $1.0 \times 10^3$  cells/well, cell aggregates with large microspheres secreted significantly larger amount of insulin than aggregates with middle microspheres or without microspheres (Fig. 6A). We got the equivalent result on the insulin secretion per cell (Fig. 6C). On the other hand, at the initial seeding density of  $1.0 \times 10^4$  cells/well, insulin secretion amount per aggregate decreased with the decrease of live cell number, and the insulin secretion amount per cell did not change with incubation time (Fig. 6B and D). It is well known that insulin secreting cells are

susceptible to a hypoxic environment and need large amount of oxygen to physiologically produce insulin [11,12]. In this connection, this result experimentally confirms that the presence of gelatin hydrogel microspheres could give INS-1 cells a larger amount of oxygen to secrete insulin. As mentioned above, it is possible that the V-bottomed well physically damaged the cell aggregates because the well bottom area is narrow compared with that of U-bottomed or flat-bottomed well. This may be explained in terms of the physical stress which greatly affects the profile of insulin secretion. The result of reductase activity was different from that of live cell number at both initial seeding densities of  $1.0 \times 10^3$  and  $1.0 \times 10^4$  cells/well, namely the activity per cell and aggregate increased with incubation time (Fig. 5). It is considered to be difficult to definitely determine the live or dead of cells by trypan blue staining method because the method can show the cell viability only by the damage degree of cell membrane. Cells detected as dead cells maintained the reductase activity and then the activity increased with incubation time. This is because the cell number increased as mentioned above. This result implies that the insulin secretion ability was sensitive to cell viability compared with reductase activity.

In this study, glucose-induced insulin secretion was evaluated as the function of insulin secreting cell aggregates because the function is the most important for the diabetes treatment. It is known that this function is damaged by the hypoxic environment most seriously. The improvement of insulin secretion ability by the microspheres is important for treatment of type 1 diabetes.

Particularly, in the present study, INS-1 cell aggregate with large gelatin hydrogel microspheres with the initial seeding density of  $1.0 \times 10^3$  cells/well would be cell aggregates prepared with the best condition for treatment of type 1 diabetes, because the aggregates secrete significantly higher amount of insulin after the same incubation time as aggregates with middle microspheres and without microspheres.

Pseudo-islets artificially formed from insulin secreting cells undergo necrosis because the cell aggregates are generally cultured under a static condition. Lock et al. or Matta et al. used the bioreactor for suspension culture to improve the oxygen and nutrients transport [31,32]. The supplementation of all-trans retinoic acid or nicotinamide to the culture medium also maintained or enhanced the functions of pseudo-islets [33–36]. Primarily isolated islets show a tendency of self-aggregation and the islet clumping often causes the serious necrosis because of the lack of oxygen and nutrients after the islet transplantation [37–39]. To tackle this issue, Liao et al. made islets separate to each other by their embedding into a hydrogel [37]. Qi et al. or Aung et al. encapsulated islets into a PVA sheet or tube not to form the clumping [38–40]. Similarly, Onoe et al. encapsulated islets into alginate fibers [41]. The gelatin hydrogel microspheres technology might be promising to avoid the necrosis under the conventional static culture condition without any devices to suppress the clumping because the microspheres function as the pathway of oxygen and nutrients in insulin secreting cell aggregates or islets. When the cell aggregate become larger, the simple supplementation to the culture medium would not reach cells in the center of aggregates sufficiently because of the inherent barrier of cell aggregates. We previously reported that gelatin hydrogel microspheres function as not only the pathway of oxygen and nutrients, but also biological factors release carrier [24,42–44]. If the supplementation of all-trans retinoic acid or nicotinamide is performed from gelatin hydrogel microspheres in cell aggregates, cells in aggregates might uniformly interact with these factors. Furthermore, it is known that primarily islets contain cells secreting vascular endothelial growth factor-A (VEGF-A) to regulate islet differentiation, form highly vascularized islets, and maintain islet functions [45–47]. It is easily predicted that the



release of VEGF from gelatin hydrogel microspheres in cell aggregates would realize the artificially formed insulin secreting cell aggregates more similar to natural islets.

The present study demonstrates that the incorporation of gelatin hydrogel microspheres at the certain size and number could improve the cell viability and insulin secretion function. This technology is promising to give insulin secreting cell aggregates a better condition for their functional improvement, although more optimization of microspheres is needed in the future.

## 5. Conclusions

INS-1 cell aggregates with or without gelatin hydrogel microspheres were formed. Cell viability and glucose-induced insulin secretion of cell aggregates were improved by the incorporation of microspheres especially at the larger size and number.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.reth.2017.12.002>.

## Conflict of interest

There is no conflict of interest to disclose.

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